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A Specific Haplotype Framework Surrounds the Omani Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Mutation S549R

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Abstract

Cystic Fibrosis (CF) is an autosomal recessive disorder affecting the chloride transport in mucus-producing epithelial cells. The disease is caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), which is responsible for trans-epithelial chloride transport. Approximately 1900 mutations and gene variants of the CFTR have been described. The spectrum of major White-European mutations includes F508del, G542X, G551D and N1303K. F508del is the most common CF-causing mutation, found in approximately 70% of all CF patients worldwide. The spectrum of CF mutations of Arab populations is under-investigated. However, initial molecular-epidemiological studies indicate the existence of specific CF mutation clusters within geographical regions in the Middle East, suggesting specific distributions of CF mutation carrying chromosomes in this part of the world. We showed that the world-wide rare CF mutation S549R is the predominant disease causing mutation in the Omani population. We reported that S549R, together with two other identified mutations, F508del and the rare private mutation V392G, are genetically linked to the exonic methionine polymorphism c.1408A>G; p.Met470Val at exon 10 and the intronic dimorphic 4-bp GATT 6-repeat at intron 6, c.744_33GATT[6_8]. We detected three haplotypes in 28 alleles of the Omani CF cohort and 408 alleles of our control cohort of unrelated and unaffected Omani volunteers. The CF disease associated haplotype consisting of an M allele and a 6-repeat expansion, occurred with an allele frequency of only 0.174 in the normal Omani population. The discriminative power of the haplotype was attributed to the intronic dimorphic 4-bp GATT 6-repeat. Furthermore, we found only one mutation, c.1733_1734delTA in the Omani CF cohort which deviated from the rule and shared the most common haplotype, a V allele and a 7-repeat extension, with the normal population.

Key words: Arab populations, Mutations, Cystic Fibrosis, Cystic Fibrosis Transmembrane Conductance Regulator, S549R, Oman, Haplotypes, Diplotypes, M470V

1. Introduction

Cystic fibrosis (CF) (OMIM 602421) is the most common autosomal recessive inherited disorder in White-European populations. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), which is a chloride channel at the apical membrane of mucus producing epithelial cells (Kerem *et al*., 1989; Riordan *et al*., 1989; Rommens *et al*., 1989). The CFTR protein is predominantly expressed in epithelial cells of the lung, pancreas and gastro-intestinal tract. Mutations in the CFTR gene and their differential impact on the number of ion channels reaching the plasma membrane, on the passage of chloride ions through the ion-conducting pathway or the amount of intrinsic ATP-driven activity of CFTR, result in altered chloride conductance and lead consequently to a multiple organ involvement in CF or CF-related disorders (Sheppard *et al*., 1996; Wang *et al*., 2014). The classical clinical CF phenotype is characterized by repeated obstruction and infection of the lung (Ciofu *et al*., 2013), a failure to thrive as a consequence of mal-digestion (pancreatic involvement) and mal-absorption (gastro-intestinal involvement) (Gelfond *et al*., 2013).

Moreover, specific mutations in this gene can contribute to a form of male infertility known as Congenital Bilateral Absence of the Vas Deference

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(CBAVD) (Daudin *et al*., 2000), idiopathic and chronic pancreatitis (Cohn, 2005; Sharer *et al*., 1998; Witt, 2003) and bronchiectasis (Feldman, 2011).

Following the cloning of the CFTR gene, detailed studies of CF causing mutations and the elucidation of mutational panels for CF in the White-European populations emerged (Bobadilla *et al*., 2002; Grody *et al*., 2001; Watson *et al*., 2004). CF has an overall prevalence of approximately 1:2500 and a carrier frequency of 1:25 in White-European populations (Bobadilla *et al*., 2002). Currently more than 1900 CF mutations and sequence variations have been reported and documented (Cystic Fibrosis Mutation Database; accessed June 2014). The major mutation in White-European populations is F508del, a deletion of phenylalanine at c.1521_1523delCTT in exon 10 which has an average frequency of approximately 70% (Bobadilla *et al*., 2002). Only four other CFTR mutations, G542X, N1303K, G551D and W1282X, have worldwide allele frequencies above 1% in White-European populations (Estivill *et al*., 1997). Therefore the majority of CFTR mutations must be considered as rare or private and their regional occurrence can be attributed to subpopulations either geographically (e.g., G551D) or religiously (e.g., W1282X) (Estivill *et al*., 1997; Bobadilla *et al*., 2002).

This situation appears to be different among Arab populations. The spectrum of CF mutations, incidence and prevalence of the disease are largely unknown in the Arab populations (Wei *et al*., 2006). However, emerging studies suggest that the major and predominant CF mutations of Arab populations are different from the known CF mutational patterns in White-European populations. To date, a predominant major CF mutation, like the F508del CF mutation in White-European populations, was not described in Arab populations. Disease causing mutations for the majority of CF cases in Arab populations are considered as rare or private in White-European and other populations. For instance, the mutation I1234V is a major mutation in Qatar (Wahab *et al*., 2001), whereas in Saudi Arabia 3120+1G>A appears to be the most common and predominant CFTR mutation (El-Harith *et al*., 1997). The CFTR mutation E1140X was reported with 40% as the most common mutation in Libya (Fredj *et al*., 2011). Another specific and distinct mutational pattern was reported for Oman (Fass *et al*., 2014) and the neighboring country, the United Arab Emirates (UAE) (Frossard *et al*., 1997). The CFTR mutation S549R, a mutation altering the signature motif of the CFTR protein, is disease causing for more than 75% of all CF cases in both Gulf countries (Fass *et al*., 2014; Frossard *et al*., 1997; Lestringant *et al*., 1999).

The CF mutational panel of Oman is defined by two major mutations S549R and F508del with frequencies in the patient cohort of 0.75 and 0.14, respectively. Other mutations, which account for the remaining allele frequency of 0.11, were private and occurred in single patients or families (Fass *et al*., 2014). Carrier screening for all identified Omani mutations of a student population at Oman Medical College confirmed clinical CF incidence data and allowed the estimation of a CF prevalence of 1:8,264 in the country (Fass *et al*., 2014).

The absence of genetic evidence for an accumulation of CFTR mutations at a specific region in the CFTR gene, a so-called hot spot, suggests different reasons for the occurrence of the large number of CFTR mutations in various populations. One of the reasons might be a selective advantage of specific variants of the CFTR gene (Pompei *et al*., 2006). It was known even before the cloning of the CFTR gene that DNA polymorphisms such as short tandem repeats and single-nucleotide polymorphisms (SNP) co-segregate with disease causing alleles (Cutting *et al*., 1984). These various polymorphic markers were used for genetic counseling, risk assessment and carrier predictions (Beaudet *et al*., 1989), and served as tools for the identification of the origin, age and evolution of CFTR mutations (Morral *et al*., 1994). Haplotype frameworks are known for common White-European CFTR mutations but remained almost completely unexplored within the specific mutation patterns of Arab populations.

Two characteristic polymorphic markers in the CFTR gene are c.1408A>G (p.Met470Val; M470V), and c.744-33GATT[6_8], which will be abbreviated henceforth as M/V and 6/7 polymorphism, respectively (Kerem *et al*., 1990; Chehab *et al*., 1991; Gasparini *et al*., 1991). The M/V polymorphism is an exonic diallelic marker in exon 10 of the CFTR gene. The M allele is known to be associated with the CFTR mutation F508del (Cuppens *et al*., 1994; Pompei *et al*., 2006). Physiological differences between the wild type (wt) V and M allelic CFTR variant exist. It has been reported that the M allele of wt-CFTR gene exerts a higher chloride conductance (Cuppens *et al*., 1998). Another more recent study by Kosova *et al*. (2010) reports that the M allele is associated with a lower birth rate in fertile man. The 6/7 polymorphism is a sequence alteration in intron 6 of the CFTR gene (Chehab *et al*., 1991; Gasparini *et al*.,1991). The 6-GATT repeat structure was reported to be associated with F508del but have remained under-explored in other non-F508del White-European CFTR mutations. A potential physiological consequence has not been reported and seems unlikely because of the distance of this sequence alteration to the splice site.

The aim of this study is to classify the initial haplotype patterns of the M/V and 6/7 polymorphisms for the recently identified Omani mutations in an Omani CF cohort and a corresponding Omani control cohort. An analysis of haplotype patterns has the potential to identify the origin of the mutation and specify the genetic risk assessment in clinical practice.

2. Materials and Methods

2.1. Omani CF Patient and Volunteer Cohort

The Omani CF patient and volunteer cohorts were described elsewhere (Fass *et al*., 2014). An informed consent was taken from the 14 unrelated CF patients and the 204 unrelated student volunteers from Oman Medical College in Sohar. Consanguinity is high in the Omani population (Rajab and Patton, 2000). We ensured, by a questionnaire, that the enrolled volunteers are not related to each other. Furthermore, we inquired about the geographic origin of the parents and the grandparents of

the volunteer. The ancestry of 172 (84.35%) volunteers could be traced over three generations to the territory of Oman. DNA was isolated from ethylene-diamine-tetra-acidic acid (EDTA) buffered blood using the Wizard® genomic DNA Purification Kit (Promega Corp., Madison, Wisconsin, USA) according to the manufactures protocol. The disease causing mutations were identified in the Omani CF cohort by analysis of the entire exonic region and the flanking introns of the CFTR gene (Fass *et al*., 2014). The following CFTR mutations occur in the CF cohort in this study: (1) S549R (c.1647T>G, p.Ser549Arg), (2) F508del (c.1521_1523delCTT, p.Phe 508del), (3) V392G (c.1175T > G, p.Val392Gly), and (4) c.1733_1734delTA with allele frequencies of 0.75, 0.14, 0.035 and 0.075, respectively. The clinical characteristics and the severity of CF for those mutations have been recently reported (Fass *et al*., 2014).

2.2. Determination of Polymorphic Loci

Two polymorphic loci M/V and 6/7 were investigated in 28 CF patients' alleles and 408 alleles of the volunteer control cohort.

The M/V polymorphism was analyzed by restriction digest with HphI (New England Bio Labs Inc, Ipswich, Massachusetts, USA) (Kerem *et al*., 1990). Briefly, PCR primer and amplification conditions were applied as described previously (Fanen *et al*., 1992). For the restriction digest 10 µL of amplification product was digested with four units of HphI in a total volume of 15 µL overnight. The restriction fragments were separated and visualized by electrophoresis of a 3% agarose gel at 90 volts for 30 minutes (Figure 1A). The 6/7 polymorphism at intron 6 was analyzed by DNA shift assay using polyacrylamide gel electrophoresis (PAGE) (Chehab *et al*., 1991). For the amplification of the 4-bp GATT repeat structure at intron 6 reported primers and PCR conditions were used (Zielinsky *et al*., 1991). 3 µL amplification products were mixed with 7µL 6X bromophenol blue / xylene cyanol-loading dye. Electro-phoresis was conducted on 10% PAGE gels (32cm x 18cm x 0.75mm), in 1X Tris-acetate-ETDA buffer (TAE) at 250 volt for 6-8 hours. The DNA shift was visualized by silver staining (Fass *et al*., 2014) (Figures. 1B and C).

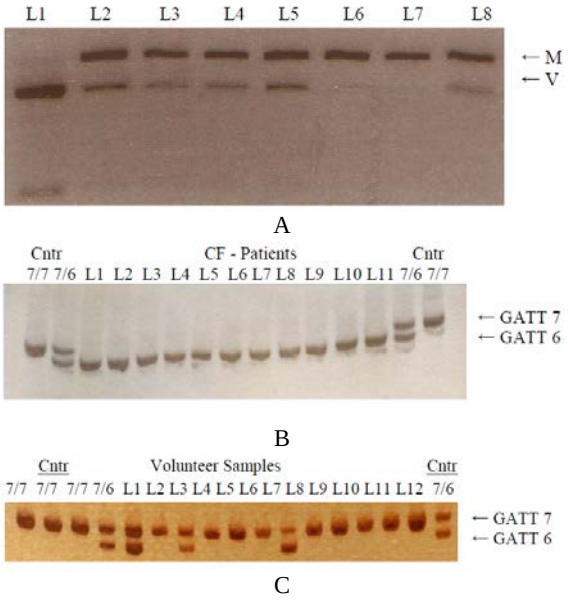


Figure 1. Representative **a**nalysis of the polymorphic loci in Omani CF patients (CF-Pts) and volunteers of the Omani Control Cohort (Vol): (A) Polymorphism M470V (M/V) of exon 10: Restriction digest with HphI on 3% agarose; The restriction site is abolished in the M allele of the M/V polymorphism. L 1: Vol= V/V (homozygote V/V); L 2-4+8: Vol=M/V (heterozygote M/V); L 6-7: Pts=M/M (homozygote M/M); (B) Dimorphic 4-bp GATT repeat polymorphism (6/7) of intron 6: DNA shift assay, 10% PAGE, silver staining: CF-Pts samples L1-9: S549R/S549R and L10-11: F508del/F508del. CF-Pts carry the 6 allele. (C) Dimorphic 4-bp GATT repeat polymorphism (6/7) of intron 6: DNA shift assay, 10% PAGE, silver staining: Vol Samples L 2, 4-6, 8-12: Vol=7/7 (homozygote 7/7), L 1,3 and 7: Vol=6/7 (heterozygote 6/7)

2.3. Analysis of Allele Frequencies, Haplotypes and Diplotypes

The allele frequencies of the polymorphic loci were established by direct allele counting. The two investigated polymorphic markers (M/V and 6/7) result in 4 theoretically possible haplotypes (M6, M7, V6, V7) and, consequently, 10 theoretically possible diplotypes (M6M6, M6V7, M6M7, M7M7, M7V7, V7V7, V7V6, V6V6, V6M7, V6M6). The Chi-square test (χ^2) was applied to compare the allele frequency differences between the Omani CF patient cohort and the Omani volunteer cohort.

3. Results

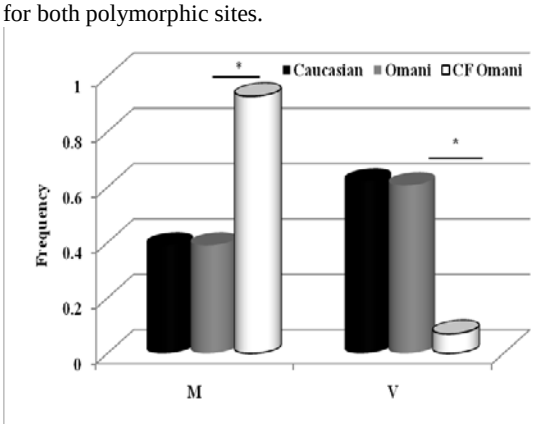
3.1. Allele frequency of M/V and 6/7 polymorphisms in the cohorts of unaffected Omani Volunteers and Omani CF patients

The analysis of M/V polymorphism and 6/7 polymorphism of the representative samples from the Omani volunteer cohort and the Omani CF patient samples are shown in Figure 1. Homozygote patients for the major Omani CF mutation S549R (allele frequency 0.75) carry a M allele at the M/V locus which results in an abolished restriction site of HphI at c.1408A (Figure 1A).

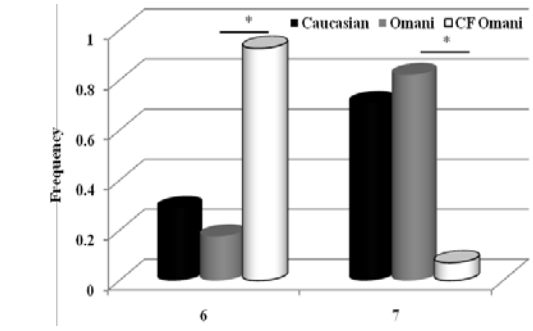
A similar pattern for the mutation S549R was observed for the 6/7 polymorphism. Patients, with the S549R CFTR mutation, are completely homozygote for the 6-GATT repeat (Figure 1B). In contrast, the 6 allele of the 6/7 polymorphism was not the most abundant allele in the Omani volunteer cohort. Figure 1C illustrates the predominance of the 7 allele in a representative electrophoresis of 12 samples from the Omani volunteer cohort.

The M/V polymorphism at exon 10 occurred in the unaffected Omani population with allele frequencies of 0.392 and 0.607, respectively (Figure 2A). In contrast, the 6/7 polymorphism at intron 6 was less polymorphic and was detected in the control cohort with allele frequencies of 0.177 and 0.822, respectively (Figure 2B).

However, the 28 investigated alleles of the CF patients revealed an almost completely different distribution of the M/V and 6/7 polymorphism. The M and 6 polymorphic makers were both associated with the Omani mutations S549R, F508del and V392G. The allele frequency for each the M and 6 polymorphism was 0.928 (Figure 2). In contrast, only the alleles of the mutation c.1733_1734delAT were associated with the V and 7 polymorphisms and were found with a frequency of 0.071 for both polymorphic sites.



A

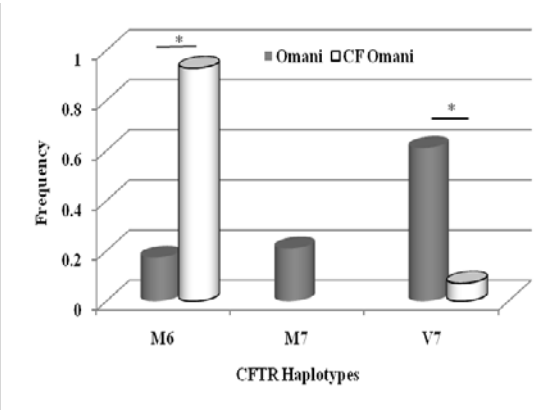


B

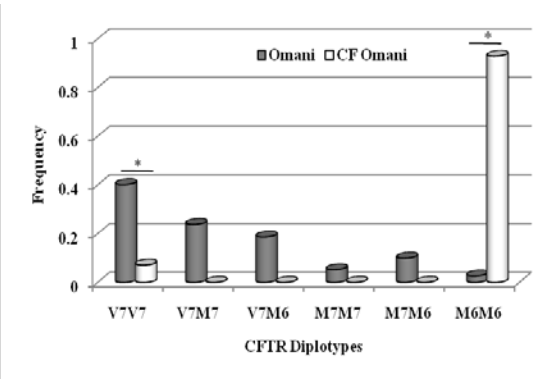
Figure 2. Allele frequencies of (A) exonic polymorphism M470V (M/V) of exon 10 and (B) intragenic, intronic dimorphic 4-bp GATT repeat (6/7) of intron 6 in White-European (Pompei F. *et al* 2006; Chehab FF. *et al.* 1991), Omani Control Cohort, and Omani CF patient population: The Omani mutations S549R, F508del and c.1175T>G are associated with the M ($\chi^2 = 3.1x10^{-8}$) and 6 ($\chi^2 = 2.1 \times 10^{-15}$) allele. Only the muation c.1733-1734 del AT is associated with V ($\chi^2 = 2.8 \times 10^{-4}$) and 7 ($\chi^2 = 1.2 \times 10^{-5}$).

3.2. Haplotypes and Diplotypes of the Omani CF and control cohorts

Out of four theoretically predicted haplotypes (M6, M7, V6, V7), only three were observed in the Omani population. The haplotype V6 was neither found in 408 alleles of the volunteer cohort nor in 24 alleles of the CF patient cohort. Therefore, only the haplotypes M6, M7 and V7 appeared to exist in the Omani population. The allele frequencies of the haplotypes M6, M7 and V7 in the volunteer cohort were 0.174, 0.214 and 0.611, respectively (Figure 3A).



A



B

Figure 3.(A) Haplotypes and (B) Diplotypes frequencies of exonic polymorphism M470V (M/V) of exon 10 and intragenic, intronic dimorphic 4-bp GATT repeat (6/7) of intron 6 in the Omani Control Cohort, and Omani CF patient population: The Omani mutations S549R, F508del and c.1175T>G are associated with the haplotype M6 ($\chi^2 = 2.8 \times 10^{-11}$) and the diplotype M6M6 ($\chi^2 = 2.0 \times 10^{-3}$). The mutation c.1733-1734 del AT appears as exception and is associated with the haplotype V7 ($\chi^2 = 2.4x10^{-5}$) and diplotype V7V7 ($\chi^2 = 4.3 \times 10^{-12}$).

Interestingly, the Omani CF mutations S549R, F508del and V392G were completely associated with the haplotype M6 ($\chi^2 = 2.8 \times 10^{-11}$). This high haplotype frequency of M6 for the mutated alleles stands in a significant contrast to the haplotype frequency of M6 in the volunteer cohort, where it was detected with an allele frequency of only 0.174 ($\chi^2 = 2.8 \times 10^{-15}$) (Figure 3A). The association of M6 implies a specific genomic polymorphic pattern surrounding the most common Omani CF mutation S549R and the other two mutations, F508del and V392G. In contrast, only the two alleles of

the mutation c.1733_1734delAT are associated with a V7 haplotype. The V7 haplotype is the most common haplotype in the Omani population (Figure 3A). Caused by the absence of the V6 haplotype in the control and patient cohort, only 6 diplotypes out of 10 possible combinations were detected. The diplotypes V7V7, V7M7 and V7M6 were with 82.5% predominance in the volunteer cohort and occurred with relative frequencies of 0.401, 0.238 and 0.186, respectively (Figure 3B). The diplotypes, M7M6, M7M7, M6M6 occurred with 17.7%, were less frequent and had relative frequencies of 0.098, 0.052 and 0.026, respectively. However, the Omani CF patients with the mutation S549R, F508del and V392G were homozygote for M and 6 and form a M6M6 diplotype. The M6M6 diplotype in the patient cohort had a relative frequency of 0.928. The test of the association between the control cohort and the patient cohort revealed a strong association of S549R, F508del and V392G with the diplotype M6M6 ($\chi^2 = 2 \times 10^{-3}$). Surprisingly, only the private mutation c.1733_1734delAT was linked to the V7V7 diplotype and had a relative frequency of 0.071. The very strong association of S549R to the haplotype M6 and, consequently, to the diplotype M6M6 revealed a core characteristic of the mutation associated polymorphic pattern in the CFTR gene which seems rare in the normal Omani population.

4. Discussion

We have previously observed, during routine diagnostic gene wide screening of all exons and intronic borders of CFTR in suspected CF-patients that the predominant Omani mutation S549R occurred together with specific polymorphisms. This initial observation prompted us to study the two polymorphic loci M/V and 6/7 in 28 alleles of Omani CF patient cohort and in 408 alleles of unrelated and unaffected Omani healthy volunteers. Our initial observation about the genetic association of specific polymorphisms with alleles carrying S549R was immediately confirmed by the analysis of the M/V and 6/ 7 loci for representative samples (Figure 1). The M and 6 allele of the M/V and 6/7 polymorphism were linked to the Omani mutation S549R. In addition, we observed that this linkage occurred to F508del and V392G (Figure 2). An exception was the private mutation c.1733_1734delTA, a recently reported CFTR mutation from Oman which caused a frameshift and a formation of a premature stop codon (Fass *et al*., 2014). This mutation was associated with a V and 7 allele. A V7 haplotype is the predominant haplotype in the volunteer cohort with an allele frequency of 0.611 (Figure 3).

The occurrence of the allele frequencies of the M/V polymorphism in the unaffected Omani population was 0.392 and 0.607, respectively. This observation is similar to the reported M/V allele frequencies in the Caucasian population where M/V was reported with allele frequencies of 0.39 and 0.62, respectively (Pompei *et al.*, 2006).

It was reported earlier that the M allele of the M/V polymorphism is associated with the CF mutation F508del (Cuppens *et al*., 1994; Dork *et al*., 1994; Ciminelli *et al*., 2007; Pompei *et al*., 2006). Therefore, the association of F508del with the M allele of the M/V polymorphism in our Omani CF cohort confirms this observation. One plausible reason for the association of F508del and other CF mutations might be a selective advantage of the V over the M allele in the CFTR gene (Pompei *et al*., 2006). Although the M/V sequence change in the CFTR gene is considered as a polymorphism, the physiological responses of both alleles appear different, which indirectly supports the notion of a selective advantage of the V allele (Cuppens *et al*., 1998; Kosova *et al*. 2010).

Furthermore, it has been reported that the association of non-F508del mutations is correlated to the genetic distance from the M allele (Ciminelli *et al*., 2007). The Omani mutation S549R in exon 11 occurs 28 kb away from the M470 site and, therefore, within a distance where a genetic association is expected (Ciminelli *et al*., 2007). However, we observed that the private mutation V392G shares similar associations with the M polymorphism. This mutation occurs in exon 8 and, therefore, is far from the M470 site.

The intronic 6-GATT repeat structure of the 6/7 polymorphism was linked to the reported Omani CF mutations S549R, F508del and V392G and had an allele frequency in the patient population of 0.928. In contrast, the allele frequency of 0.177 of the 6 polymorphism in the volunteer cohort was a rare genetic manifestation. This means that the 6 polymorphism is by 42% less frequent in the Omani volunteers in comparison to the reported frequency of 0.29 in White-European populations (Chehab *et al.*, 1991) (Figure 2). Its low allele frequency provides a discriminative power for a potential risk assessment of the genetic predisposition of a disease causing CFTR mutation within the Omani population. Due to the absence of a V6 haplotype, the M6 haplotype appears to define a CF disease predisposition in Oman. The allele frequency of 0.17 for M6 represents 35 unaffected healthy individuals of the Omani control cohort. This means that those 35 individuals (70 alleles) are on higher risk of being CF carriers, whereas other volunteers have a reduced risk. We found two CF carriers in 204 individuals, one carrier with S549R and another with F508del (Fass *et al*., 2014). Both CF carriers had a M6 haplotype which highlights the clinical genetic significance of haplotypes as additional CFTR screening tools. It is possible that other, yet unidentified polymorphisms or haplotypes are associated with an even larger discriminative power to S549R and other Omani mutations. A further exploration and extension of haplotype pattern in the Omani population would clarify the origin and character of the rare mutation c.1733_1734delTA which shared the V7 haplotype.

5. Conclusion

We demonstrated that the major Omani CFTR mutation S549R, together with two other identified CFTR mutations in the Omani population, F508del and V392G, are associated with the haplotype M6 of the polymorphic markers M/V and 6/7. M6 is a rare haplotype in the Omani population, suggesting a migration of this

haplotype into the population. A fourth, recently identified CFTR mutation in Oman, c.1733_1734delTA, was associated with the haplotype V7. The highest haplotype frequency in the unaffected Omani population is V7. Although the association of the haplotype V7 with CF appeared like an exception, there is the possibility that this rare mutation originates from Oman.

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